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Experimental acquisition, development, and transmission of *Leishmania tropica* by *Phlebotomus duboscqi*

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ABSTRACT

We report experimental infection and transmission of *Leishmania tropica* (Wright), by the blood-feeding sand fly *Phlebotomus duboscqi* (Neveu-Lemaire). Groups of laboratory-reared female sand flies that fed “naturally” on *L. tropica*-infected hamsters, or artificially, via membrane feeding device, on a suspension of *L. tropica* amastigotes, were dissected at progressive time points post-feeding. Acquisition, retention and development of *L. tropica* through procyclic, nectomonad, and leptomonad stages to the infective metacyclic promastigote stage, and anterior progression of the parasites from abdominal midgut bloodmeal to the thoracic midgut were demonstrated in both groups. Membrane feeding on the concentrated amastigote suspension led to metacyclic promastigote infections in 60% of sand flies, whereas only 3% of *P. duboscqi* that fed naturally on an infected hamster developed metacyclics. Sand flies from both groups re-fed on naïve hamsters, but despite infections in 25–50% of membrane-fed and 2–3.5% of naturally fed flies, no skin lesions developed in the hamsters. After four months of observation these animals were euthanized and necropsied. Screening of the organs and tissue by polymerase chain reaction (PCR) that targeted the small subunit RNA gene, amplified generic *Leishmania* DNA from liver, spleen, bone marrow, and blood, but only from hamsters bitten by membrane-infected *P. duboscqi*. These results are notable in demonstrating the ability of *P. duboscqi*, originating from Kenya, to acquire, retain, develop, and transmit a Turkish strain of *L. tropica* originally isolated from a human case of cutaneous leishmaniasis. This marks the first demonstration of complete development and transmission of *L. tropica* by a member of the *Phlebotomus* subgenus of sand flies.

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1. Introduction

Leishmania tropica is a primary causal agent of Old World cutaneous leishmaniasis (CL), but this parasite is unusual among all others in the Old World *Leishmania* complex because of clinical presentations that range from simple, localized dermatological lesions to disseminated visceral leishmaniasis, and even oro-nasal and naso-pharyngeal leishmaniasis (Schnur, 1989; Magill et al., 1993; Sacks et al., 1995). While regarded largely as an anthroponotic disease with man as the reservoir of infection (Ashford et al., 1992; Killick-Kendrick et al., 1995; Jacobson, 2003), *L. tropica* has been repeatedly isolated from the rock hyrax (*Procavia capensis*) in Israel and Kenya, and from domestic dogs in Morocco (Dereure et al., 1991; Sang et al., 1994). Hamsters, black rats, and inbred mice can be infected experimentally but intensive investigations at foci of disease in Israel have failed to identify natural infections in other small mammals (Bastien and Killick-Kendrick, 1992; Svobodová et al., 2006; Jacobson, 2003). Recently, however, two isolates of *L. tropica* were reported from wild *Gerbillus pyramidum* collected in the north Sinai from a site near Raffah considered a “classical focus of *Leishmania major*” (Shehata et al., 2009) and PCR screening of wild golden jackals (*Canis aureus*) and red foxes (*Vulpes vulpes*) captured from sites throughout Israel detected *L. tropica* from ears and spleens in 8% of these animals (Talmi-Frank et al., 2010). Foci of *L. tropica* infection occur sporadically across North Africa and as far south as Namibia, then northward through Kenya and Ethiopia and into the Arabian Peninsula (Gebre-Michael et al., 2004). Throughout its range in North Africa, the Eastern Mediterranean, and Western Asia, *Phlebotomus sergenti* is the most common Old World vector of *L. tropica*. *Phlebotomus guggisbergi* is the primary vector of *L. tropica* in Kenya (Lawyer et al., 1991), and *Phlebotomus arabicus* has

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14. ABSTRACT

We report experimental infection and transmission of Leishmania tropica (Wright), by the blood-feeding sand fly Phlebotomus duboscqi (Neveu-Lemaire). Groups of laboratory-reared female sand flies that fed ?naturally? on L. tropica-infected hamsters, or artificially, via membrane feeding device, on a suspension of L. tropica amastigotes, were dissected at progressive time points post-feeding. Acquisition, retention and development of L. tropica through procyclic, nectomonad, and leptomonad stages to the infective metacyclic promastigote stage, and anterior progression of the parasites from abdominal midgut bloodmeal to the thoracic midgut were demonstrated in both groups. Membrane feeding on the concentrated amastigote suspension led to metacyclic promastigote infections in 60% of sand flies, whereas only 3% of P. duboscqi that fed naturally on an infected hamster developed metacyclics. Sand flies from both groups re-fed on na?ve hamsters, but despite infections in 25?50% of membrane-fed and 2?3.5% of naturally fed flies, no skin lesions developed in the hamsters. After four months of observation these animals were euthanized and necropsied. Screening of the organs and tissue by polymerase chain reaction (PCR) that targeted the small subunit RNA gene, amplified generic Leishmania DNA from liver, spleen, bone marrow and blood, but only from hamsters bitten by membrane-infected P. duboscqi. These results are notable in demonstrating the ability of P. duboscqi, originating from Kenya, to acquire, retain, develop, and transmit a Turkish strain of L. tropica originally isolated from a human case of cutaneous leishmaniasis. This marks the first demonstration of complete development and transmission of L. tropica by a member of the Phlebotomus subgenus of sand flies.

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recently been confirmed as a vector in Israel (Jacobson et al., 2003). Other confirmed and putative vectors include *Phlebotomus saevus* (Gebre-Michael et al., 2004), *P. aculeatus* (Johnson et al., 1993), and *P. rossi* (Rioux et al., 1990). Experimentally, *Phlebotomus halapensis*, *P. argentipes*, and even the New World sand fly, *Lutzyomia longipalpis* have been found to support the growth and differentiation of *L. tropica*, as well as *L. major*, earning them the title “permissive” (Sadlova et al., 2003; Kamhawi, 2006).

Phlebotomus duboscqi, a member of the *Phlebotomus* subgenus, is an important anthropophilic sand fly found throughout the Sub-Saharan northern half of Africa, from Senegal to Ethiopia, south to Kenya and into the Arabian Peninsula (Lewis, 1982; Killick-Kendrick, 1990). It is a confirmed vector of *L. major* in Senegal, Kenya, and Ethiopia, and is considered to be the primary vector of *L. major* across the Sahel region of Africa (Dedet et al., 1982; Beach et al., 1984; Killick-Kendrick, 1990). The role of *P. duboscqi* in transmitting *L. tropica* is unknown but in comparison with *Phlebotomus papatasi*, a closely related member of the *Phlebotomus* subgenus, *P. duboscqi* demonstrated biting behavior, longevity, gonotrophic discordance, and infection levels that made it a “much more effective vector” (Mukhopadhyay and Ghosh, 1999). Interestingly, these two species are genetically close enough to hybridize (Ghosh et al., 1999), but while *P. duboscqi* has shown an “unrestricted” capacity to support metacyclogenesis of both *L. tropica* and *L. major* (Killick-Kendrick et al., 1994), *P. papatasi* is unable to support the growth of any other *Leishmania* species but that which it transmits in nature (Sacks and Kamhawi, 2001; Kamhawi, 2006). The unusual genetic heterogeneity of *L. tropica*, its extremely wide geographical range throughout Africa, and its progression into Western Asia may be indicative of this parasite’s ability to parasitize and develop within a broad range of mammalian-feeding sand fly species (Schwenkenbecher et al., 2005; Pratlong et al., 2009). Additional support for this concept derives from the fact that natural infections of *L. tropica* have been reported, thus far, in *Paraphlebotomus*, *Larroussius*, *Synphlebotomus*, and *Adlerius* subgenera (Jacobson, 2003; Gebre-Michael et al., 2004). We sought to test this potential further and to challenge the vectorial capability of *P. duboscqi* by deliberately pairing a Turkish strain of *L. tropica* with a Kenyan strain of *P. duboscqi*. If successful acquisition, growth, differentiation, and transmission resulted from this unlikely, unnatural combination, then such an outcome might be taken as further indication of *L. tropica*’s unique adaptability and capacity for survival.

2. Materials and methods

2.1. Sand flies

Sand flies used in this study came from a colony of *P. duboscqi* established in Kenya and maintained at the Walter Reed Army Institute of Research, Silver Spring, MD, USA. The colony was in its F54 generation. Colonization and maintenance procedures were similar to those of Modi and Tesh (1983), with larvae and adults held at 27 °C, 90% relative humidity, and a 12:12 h light:dark photoperiod. Adult flies were fed 30% sugar water. To encourage blood-feeding, females were fasted for 24 h. Female *P. duboscqi* were 3–4 days old at first blood-feeding.

2.2. Host/reservoir mammals

Adult Syrian golden hamsters (*Mesocricetus auratus*) were used in these experiments. All animals were cared for and maintained under conditions approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The research plan was reviewed and approved by a Scientific Review Board and a duly constituted Institutional Animal Care and Use Committee (IACUC). Hamsters were anesthetized with a mixture of ketamine–xylazine–acepromazine (ketamine 50 mg/kg, xylazine 5 mg/kg, and acepromazine 0.5 mg/kg) given by intraperitoneal injection. Euthanasia was by CO_2 asphyxia.

2.3. Parasites

L. tropica (MHOM/TR/05/EP119) was isolated in 2005 from a CL patient in Ayden City, Turkey. The isolate was propagated in NNN medium and log-phase cultured promastigotes were cryopreserved in liquid nitrogen. For experiments, promastigotes were thawed, inoculated into Schneider’s *Drosophila* medium and grown to stationary phase. A 27-gauge needle was used to inoculate 25 μl of media containing 7×10^6 to 7×10^7 parasites/ml into the left hind foot of hamsters. *Leishmania* lesions appeared within 25–30 days post-inoculation (Fig. 1). Infected hamsters with foot lesions were used for direct natural feeding by sand flies. Subsequently these infected animals were euthanized and the lesion tissue was extirpated and homogenized in a glass tissue grinder containing 1 ml of sterile phosphate-buffered saline (PBS).

2.4. Sand fly infection by membrane feeding device

The homogenate of tissue amastigotes in PBS was mixed with an equal volume of hamster blood to produce an amastigote suspension for membrane feeding of sand flies. A taut 1-day-old chick skin membrane was used with a glass water-jacketed feeding bell for blood-feeding (Tesh and Modi, 1984). Groups of 3–4 days old female sand flies were allowed to feed for 2 h on the warm amastigote suspension. Blood-engorged flies were counted, transferred to screened cages, and held under colony conditions. Randomly selected, blood-fed flies were dissected in PBS at 16 and 20 h, then on days 1–9 post-feeding to determine parasite acquisition and the progress of infections. Alimentary tracts of each sand fly were examined via light microscopy at 40× magnification. The location, developmental stage, and approximate density of *Leishmania* parasites were recorded for each sand fly. Developmental stages of *L. tropica* were determined by morphology, motility, and position (Lawyer et al., 1990; Pimenta et al., 1992; Gossage et al., 2003) from fresh dissections. Midgut infection density at each time point was scored as 1+ (<100 parasites), 2+ (100–500 parasites), 3+ (500–1000 parasites) or 4+ (>1000 parasites) (Chiakova and Volf, 1997).

2.5. Transmission by bite from infected sand flies to naïve hamsters

Infected sand flies were allowed to re-feed on anesthetized naïve hamsters 8–9 days after feeding by membrane on *L. tropica* amastigote suspensions or 8–9 days after direct feeding on the swollen foot of an infected hamster. Blood-engorged and unfed flies were dissected immediately after re-feeding to determine their infectivity and to score infections. Hamsters were monitored over 18–19 weeks for signs of infection after which they were euthanized and necropsied. Blood, spleen, liver, and bone marrow from each animal were separately preserved at −20 °C for later analysis.

2.6. Sand fly infection by direct feeding on an infected hamster

The ability of *P. duboscqi* to acquire *L. tropica* amastigotes via natural blood feeding from an infected hamster, mature these parasites to infective stage, and transmit the infection to a naïve hamster was studied. Groups of 3–4 days old, fasted female flies were allowed to feed for 2 h directly on foot lesions of anesthetized hamsters. Blood-engorged flies were counted, returned to cages and held under the colony conditions. Representative groups of these flies were

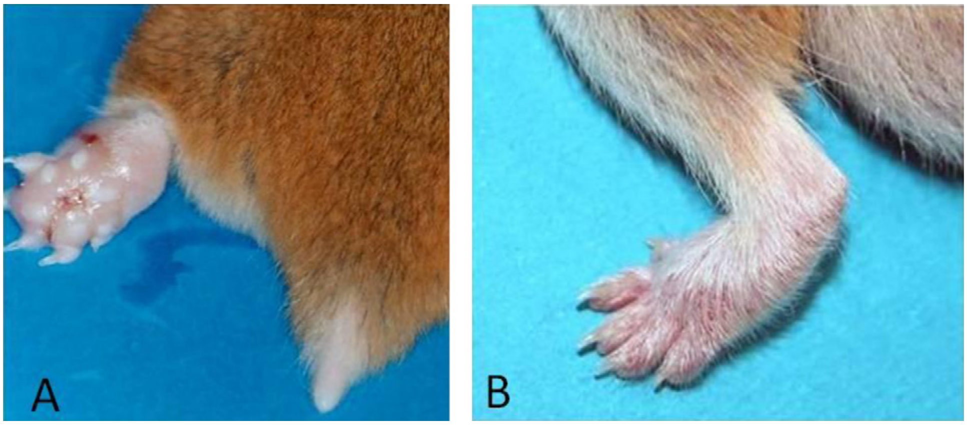

Fig. 1. (A) *Leishmania tropica* strain MHOM/TR/05/EP119 lesion in the left hind foot of a Syrian golden hamster 25 days post infection. (B) Uninfected control.

dissected 5 days post-feeding to confirm the presence, quantity, stage and location of parasites using the criteria described above for membrane-fed sand flies. Flies surviving to day 11 were fasted for 24 h and allowed to re-feed on naïve hamsters, after which both fed and unfed flies were dissected to determine and quantify their infectivity. Hamsters were monitored for signs of infection as described above.

2.7. *Molecular detection of Leishmania DNA by polymerase chain reaction*

Total DNA was extracted from hamster tissue/organ samples and from the cultured *L. tropica* reference strain (MHOM/TR/05/EP119) using the Qiagen DNA Mini Kit (Qiagen, Valencia, CA). Tissue/organ samples were macerated, homogenized in a glass tissue grinder with sterile PBS and DNA was extracted according to manufacturer's instructions. Extracted DNA was stored at −20 °C. Real-time PCR (RT-PCR) analysis was performed as described by Villinski et al. (2008) using a generic primer set for *Leishmania* spp. targeting the highly conserved small-subunit ribosomal RNA gene (LEIS.U1: 5′-AAGTGCTTTCCCATCGCAACT-3′, LEIS.L1: 5′-GACGCACTAAACCCCTCCAA-3′, LEIS.P1: 5′-[DFAM]CGGTTCGGTGTGTGGCGCC[DTAM]-3′). Real-time PCR analyses were first performed on the Cepheid Smart Cycler II (Cepheid, Sunnyvale, CA) platform in a 26 μl final volume using Cepheid SmartMix-HM PCR beads (SMHMI100-N-200) for final reaction conditions of 3 U polymerase, 200 μM/L dNTPs, 4 mM/L $MgCl_2$, 4.25 mM/L HEPES buffer (pH 7.2), 800 nM/L of each primer, and 120 nM/L dual labeled TaqMan probe (Sigma-Genosys Corp., The Woodlands, TX). Probes were synthesized with 6-FAM as the 5′ reporter and TAMRA as the 3′ quencher. For most experiments, 2 μl of DNA template was added to the reaction tube, and each run included at least one positive control (2 μl of *L. tropica* reference strain DNA) and one negative control (2 μl of nuclease-free water). Thermal Cycler conditions were as follows: step 1: 95 °C, 120 s; step 2: 95 °C, 15 s; step 3: 60 °C, 32 s; repeat steps 2 and 3 for 40 cycles. Results were confirmed on an Applied Biosystems 7500/7500 Fast (Applied Biosystems, Foster City, CA) platform in a 26 μl final volume using Go Taq® Flexi DNA Polymerase (Promega, Madison, WI). Final reaction conditions were 5.2 μl colorless Go Taq® Flexi DNA Polymerase, 3 U polymerase, 200 pM/L dNTPs, 2 mM/L $MgCl_2$, 800 nM/L each primer, 120 nM/L dual labeled TaqMan probe, using the same generic *Leishmania* primer-probe set and thermal cycling conditions.

3. Results

3.1. *Infection of sand flies by membrane feeding*

Engorgement rates in three separate trials of sand flies fed via membrane apparatus on amastigote-blood suspensions ranged from 25 to 55% and averaged 38% overall. Out of 150 suspension-fed flies, 92 were dissected at different time points post-feeding and 48 (52%) were found to harbor *L. tropica* parasites in various stages of promastigote development (Table 1). Mortality of suspension-fed flies during the 10 days following membrane feeding was 23%. Light to moderate infections by non-motile, flagellated procyclic and elongated motile nectomonad forms of *L. tropica* were observed in sand fly midguts 16–20 h post-feeding with large numbers of nectomonad stage promastigotes in the abdominal midgut through

Table 1
Infection rates and intensity of *L. tropica* promastigotes in the gut of *P. duboscqi* (after membrane feeding on a suspension of amastigotes).

Time post-infection	No. infected/total dissected	Midgut infection score[a]			Parasite stages and location
		1+	2+	3+	
16 h	5/5	5			Procyclics inside bloodmeal
20 h	5/6	2	3		Procyclics and nectomonads inside bloodmeal
1 day	4/5	1	2	1	Procyclics and different sizes of nectomonads inside bloodmeal
2 days	4/6	1		3	Procyclics and nectomonads inside bloodmeal
3 days	1/5	1			Procyclics and nectomonads inside bloodmeal and posterior midgut
4 days	2/5	2			Nectomonads and leptomonads, in anterior and posterior midgut
5 days	5/5	1	1	3	Nectomonads, leptomonads, and metacyclics in anterior and posterior midgut
6 days	3/5			3	Nectomonads, leptomonads and metacyclics in anterior midgut
7 days	2/5		2		Nectomonads, leptomonads and metacyclics in anterior midgut
8 days	7/17	2	2	3	Nectomonads, leptomonads and metacyclics in anterior midgut
9 days	2/8	2			Nectomonads, leptomonads and metacyclics in anterior midgut

[a] 1+: light (<100 parasites/fly); 2+: moderate (100–500 parasites/fly); 3+: heavy (500–1000 parasites/fly).

Table 2
Transmission of *Leishmania tropica* from infected *Phlebotomus duboscqi* to naïve hamsters.

Trial[a]	Days from initial infection to re-feeding	No. flies re-feeding (%)	No. re-feeding flies infected (%)	Hamster tissues positive for *L. tropica* by PCR
1	9	8/15 (53.3)	2/8 (25.0)	Liver, spleen, bone marrow and blood
2	8	12/29 (41.4)	6/12 (50.0)	Liver and bone marrow
3	11	57/70 (81.4)	2/57 (3.5)	None
4	11	51/55 (92.7)	1/51 (1.9)	None

[a] Hamster re-feedings 1 and 2 used flies initially infected by membrane feeding; hamster re-feedings 3 and 4 used flies initially infected by natural feeding on foot lesion.

days 5, 6 and 8. Following bloodmeal digestion, 3–5 days post blood-feeding, low numbers (1–4/microscopic field) of metacyclic promastigotes were observed in the anterior end of the thoracic midgut through day 9 post-feeding. No parasites were observed in the head, pharynx, salivary glands, or mouthparts of any fly that was dissected.

3.2. Natural infection of sand flies

In contrast to feeding rates <50% for *P. duboscqi* fed via membrane on amastigote suspensions, all flies that fed directly on the foot lesions of *L. tropica*-infected hamsters engorged fully. Samples of these naturally fed flies that were dissected 5 and 11 days after engorgement found that relatively few had acquired, or successfully retained and matured *L. tropica* amastigotes from the infected lesion. Mortality rates by day 11 for blood-engorged *P. duboscqi* in two separate feeding trials were 62.4% (78/125) and 57.7% (75/130). Despite a 16.7% infection rate on day 5 post-blood-feeding, dissections on day 11 at the time of re-feeding identified metacyclic stage promastigote infections in only 3.5% (2/57) and 1.8% (1/55) of surviving flies in the two feeding groups. Metacyclic promastigote infections were confined to the anterior thoracic midgut and were all graded as 1+ (<100 promastigotes).

3.3. Transmission/transfection trials

P. duboscqi that initially fed by membrane on amastigote suspensions demonstrated natural re-feeding rates of 41% (12/29) and 53% (8/15) on naïve hamsters compared to re-feeding rates of 81% (57/70) and 93% (51/55) for flies that had been initially infected by direct feeding on foot lesions (Table 2). Dissections revealed that 8 of 20 (40%) re-feeding membrane-fed flies were infected with promastigote infections graded as 1+ (<100 parasites) to 3+ (500–1000 parasites). No head infections were observed in any of the flies that re-fed. No cutaneous lesions developed on the two hamsters during 18 weeks of observation. These animals were euthanized, necropsied, and the blood, bone marrow, and organs were tested by RT-PCR for the presence of *Leishmania* DNA. Table 2 shows that the generic primers, targeting the small subunit RNA gene of *Leishmania*, amplified target DNA from liver and bone marrow of both hamsters that were bitten by groups of membrane-infected *P. duboscqi*. *Leishmania* DNA was also amplified from the blood and spleen from one of these animals. Two re-feeding trials with flies that had been initially infected by direct feeding on the lesion of an infected hamster demonstrated high re-feeding rates, but light, grade 1+ metacyclic promastigote infections in only 3 of the 106 (2.8%) re-feeding flies. No signs of infection were seen over four months of observation in the two naïve hamsters they bit and no *Leishmania* DNA was detected in tissue or organs from these animals (Table 2).

4. Discussion

These feeding studies demonstrated that a laboratory colony of *P. duboscqi*, originating from Kenya, was susceptible to infection by a strain of *L. tropica* from Turkey and supported its development from amastigote to metacyclic stage promastigotes that were transmitted by bite. Demonstration that naturally or experimentally infected sand flies can maintain *Leishmania* infection throughout the extrinsic life cycle of the parasite is essential for vector incrimination and the experimental transmission of *Leishmania* spp. via sand fly bite is generally considered to be conclusive evidence that a sand fly species can vector a given parasite (Killick-Kendrick and Ward, 1981; Lawyer et al., 1990). The observed development and propagation of *L. tropica* in *P. duboscqi* were typically suprapylarian, and consistent with descriptions for proven vectors of this parasite (Sang et al., 1992; Lawyer et al., 1991; Kamhawi et al., 2000). Notably in our experiments the ingested amastigotes rapidly morphed to the procyclic stage and manifested in each of the four recognized promastigote forms (nectomonad, leptomonad, haptomonad, and metacyclic promastigote) including the stage associated with transmission and mammalian infectivity. Procyclics developed within the peritrophic matrix, proliferated, and survived the digestion process. After digestive breakdown of the peritrophic matrix, nectomonads infected the abdominal midgut, attached to the epithelium and migrated forward as leptomonads into the anterior thoracic midgut. Infection of the stomodeal region of the thoracic midgut by a mixed population of active metacyclic and non-motile haptomonad stages developed in each of three separate groups of *P. duboscqi* that were fed via membrane on a concentrated suspension of amastigotes, but in just 3% of flies that fed naturally on hamster foot lesions. This difference in infection rate was expected and considered to be the dose-related outcome of feeding on a concentrated suspension of amastigotes. The 3% metacyclic promastigote infection rate achieved by direct feeding in *P. duboscqi* was not appreciably lower than natural infections of *L. tropica* in Israel by *P. sergenti* and *P. arabicus* (Jacobson, 2003), or by *P. guggisbergi* in Kenya (Lawyer et al., 1991; Johnson et al., 1999). Notably, the golden hamster is strictly an experimental host and ingestion of *L. tropica*-infected macrophages by biting flies may be rare in this animal model (Killick-Kendrick, 1990). It should also be remembered that a strain of *L. tropica* from Kabul, Afghanistan, fed as an amastigote suspension, produced a 70% infection rate in a Senegalese strain of *P. duboscqi* compared with just a 6% infection rate in the local, Kabul strain of *P. papatasi* (Killick-Kendrick et al., 1994). It seems surprising that while *P. duboscqi* and *P. papatasi* are genetically close enough to hybridize, their vectorial capacities should be quite different: the former an excellent life support system for *L. tropica* from very diverse geographic locations, but the latter limited to *L. major*, and reportedly so restricted that it cannot support non-local strains of this parasite (Kamhawi et al., 2004). The important message is that despite wide geographic and evolutionary divergence, and defying coincidence, *P. duboscqi* appears to present functional binding sites on its midgut epithelium for parasite lipophosphoglycan (LPG) that were essential, and sufficient for recognition, attachment, and development by Middle Eastern strains of *L. tropica* (Sacks et al., 2000; Kamhawi et al., 2000; Lig, 2001; Soares et al., 2004).

In two separate experiments, transmission of *L. tropica* by *P. duboscqi* was demonstrated by detection of parasite DNA in hamsters four months after biting. Both transfections resulted from bites by flies that had been initially infected by membrane feeding on a concentration of amastigotes that may far exceed the number of parasites acquired by natural feeding on an infected host (Yuval, 1991). We did not observe *L. tropica* parasites in the pharynx or mouthparts of flies infected by either membrane or natural feeding and assume that metacyclics were passed from the sand flies into hamsters as a result of stomodeal blockage and reflux during the act of blood feeding (Jefferies et al., 1986; Schlein et al., 1992; Volf et al., 2004). It is unusual that no signs of cutaneous pathology developed in either of two separate trials where *L. tropica* had been transmitted by membrane-infected *P. duboscqi*, particularly since the re-feeding flies had relatively high infection rates and heavy, grade 3+ (500–1000/fly) promastigote densities in about half (9/19) of these infections. Furthermore, since reasonable numbers of membrane-infected flies did re-feed, their engorgement was taken to indicate that parasite reflux into the host had presumably occurred. The failure of these transfections to initiate pathology within four months of biting may be due to insufficient parasite numbers transferred or to a reduced affinity, or ability, of the parasites that developed in this sand fly species. Another possibility is that a critical biochemical component of the insect's saliva, the LPG, or the promastigote-induced secretory gel was missing or dysfunctional (Kamhawi et al., 2000; Rogers et al., 2002, 2004; Spath, 2003). This same *L. tropica* strain produced cutaneous lesions and disseminated visceral infection when mechanically inoculated into BALB/c mice (Mahmoudzadeh-Niknam et al., 2007) and routinely produced cutaneous lesions and visceral infections in our laboratory hamsters. However, despite its sand fly origin, it had not, since isolation, been passed through a permissive sand fly, and it is possible that some type of "conditioning" may have been a factor for local, cutaneous pathology at the inoculation site (Bates, 2007; Kamhawi, 2006). However few or altered these parasites may have been after development and passage through *P. duboscqi*, their presence after four months in the liver, spleen, bone marrow, and blood of robustly healthy laboratory hamsters attests to their critical competence in exiting the sand fly vector, gaining entry to the bloodstream of a warm blooded mammal, and then evading destruction, multiplying, and distributing widely within that mammalian host. Notwithstanding an absence of pathology or a marginally competent vector, the location-specific presence of parasite DNA in bone marrow and blood months after introduction by sand fly bite is possibly the key measure of fitness and potential for transmission of any *Leishmania* species. This insidious aspect of *L. tropica*, which may underlie its successful anthroponotic occurrence, enables the parasite to create long-term, "silent" reservoirs of infection. Such a tendency, combined with successful acquisition and development in a number of different sand fly vectors, raises the possibility that human and zoonotic infections of *L. tropica* are more common than is currently assumed from clinical presentations.

Also of note, we observed a marked preference and avidity of *P. duboscqi* for direct, natural blood feeding, and re-feeding over artificial membrane feeding that was unexpectedly offset by higher, rapid mortality in flies that had fed naturally. *Leishmania* are generally not credited with a significant lethal effect upon their sand fly hosts but it was suspected that membrane feeding a concentrated suspension of *L. major* amastigotes to *P. papatasi* led to an unnaturally heavy infection that reduced its survival (Hanafi et al., 2011).

In summary, the findings demonstrated that *P. duboscqi* was able to: (1) acquire *L. tropica* naturally, by blood-feeding on an infected mammal, (2) retain this parasite through the process of blood digestion, (3) support its multiplication and development to infective stage, and (4) successfully transmit the infective stage of *L. tropica* via natural blood-feeding. These demonstrations fulfill three of the four criteria proposed by Killick-Kendrick (1990, 1999) for incrimination of a vector: (1) the sand fly supports development of the parasite after the infecting blood-meal has been digested and defecated, (2) the sand fly demonstrates obligate, natural blood-feeding across a broad range of mammalian hosts, including man, and (3) the fly is able to transmit the parasite to a living host via bite. The fourth criterion for vector incrimination – isolation of the parasite from wild-caught sand flies– now seems possible at locations in Africa and the Arabian Peninsula where the geographic occurrences of *L. tropica* and *P. duboscqi* overlap.

Authors' disclosure statement

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, or the U.S. Government. One co-author is a military service member, the first author and other co-authors are employees of the U.S. Government. This work was prepared as part of our official duties. Title 17 U.S.C. §105 provides that 'Copyright protection under this title is not available for any work of the United States Government'. Title 17 U.S.C. §101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person's official duties.

The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals", Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996. The research protocol and methods employed in this work were reviewed and approved by a duly constituted Institutional Animal Care and Use Committee (IACUC), NAMRU-3 Animal Use Protocol No. 08-01. All animals used in this work were cared for and maintained under conditions approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). This work was supported by NMRDC, Bethesda, MD, Work Unit Number 00101.BUX.3408.

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